SUBSTITUTE FORM PTO-1390 (REV 5-93)

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
8830-10 (157952)

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)
10/009583

EXPRESS MAIL NO. EL813776917

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB00/01753	**08 May 2000**	**07 May 1999**

TITLE OF INVENTION: **Pigment**

APPLICANT(S) FOR DO/EO/US: **Buttle, Louise Georgina**

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. _X_ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ____ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. _X_ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371 (b) and PCT Articles 22 and 39(1).

4. _X_ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. _X_ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. _X_ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ___ has been transmitted by the International Bureau (as noted in PCT/IB/308).
   c. ___ is not required, as the application was filed in the United States Receiving Office (RO/US).

6. ___ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. _X_ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. __ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. __ have been transmitted by the International Bureau.
   c. __ have not been made; however, the time limit for making such amendments has NOT expired
   d. _X_ have not been made and will not be made.

8. _X_ Amendments to the claims of the International Application under PCT Article 34.
   a. _X_ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ___ have been transmitted by the International Bureau (as noted in PCT/IPEA/416).
   c. ___ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ___ have not been made and will not be made.

9. ___ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

10. _X_ A copy of the unsigned oath or declaration of the inventors. (35 U.S.C. 371 (c)(4)).

11. ___ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 12. to 16. below concern other document(s) or information included:

12. _X_ An information Disclosure Statement under 37 CFR 1.97 and 1.98.

13. ___ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. _X_ A FIRST preliminary amendment.
    ___ A SECOND or SUBSEQUENT preliminary amendment.

15. ___ A substitute specification.

16. ___ A change of power of attorney and/or address letter.

17. _X_ Other items or information:
    Certificate of Express Mailing Under 37 CFR 1.10

JC07 Rec'd PCT/PTO 06 NOV 2001

U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER:
10/009583	PCT/GB00/01753	32076-157952

18. X The following fees are submitted:	CALCULATIONS	PTO USE ONLY
Basic National Fee (27 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO **$890.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482)		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)		
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$ 890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than __ 20 X 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	**$ 130.00**	

Claims	Number Filed	Number Extra	Rate		
Total Claims	11- 20 =	0	x $18.00	**$ 0.00**	
Independent Claims	3 - 3 =	0	x $84.00	**$ 0.00**	
Multiple dependent claim(s) (if applicable)			+ $280.00	**$ 0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$ 1020.00**	
Reduction by 1/2 for filing by small entity, applicable. Applicant is a Small Entity.				**$ 0.00**	
SUBTOTAL =				**$ 1020.00**	
Processing fee of **$130.00** for furnishing the English translation later than __ 20 __ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				**$ 0.00**	
TOTAL NATIONAL FEE =				**$ 1020.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				**$ 0.00**	
TOTAL FEES ENCLOSED =				**$ 1020.00**	
				Amount to be refunded	**$**
				Amount to be charged	**$ 1020.00**

a. X A check in the amount of **$ 1020.00** to cover the above fees is enclosed.

b. ___ Please charge my Deposit Account No. _____ in the amount of $___ to cover the above fee. A duplicate copy of this sheet is enclosed.

c. X The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **500573**. A duplicate of this sheet is enclosed.

NOTE: Where an appropriate time limit under 367 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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REGISTRATION NUMBER

PHIP\310864\1

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
NATIONAL STAGE

In re:	Patent application of Buttle, Louise G.	International Application No.: PCT/GB00/01753
		International Filing Date: 8 May 2000
Serial	No.: Not Yet Assigned	Group Art Unit: Not Yet Assigned:
Filed:	Concurrently Herewith	Examiner: Not Yet Assigned.
For:	PIGMENT	Attorney Docket: 8830-10 (157952)

PRELIMINARY AMENDMENT

Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

Prior to examination of this application and before calculation of the filing fee, please amend the application, without prejudice, in accordance with the following.

Charge any fee or credit any overage associated with this preliminary amendment or the application filing to Deposit Account No. 500573.

CERTIFICATE OF MAILING
UNDER 37 C.F.R. 1.10

EXPRESS MAIL Mailing Label Number: EL 813776917
Date of Deposit: 11/6/01

I hereby certify that this correspondence, along with any paper referred to as being attached or enclosed, and/or fee, is being deposited with the United States Postal Service, "EXPRESS MAIL–POST OFFICE TO ADDRESSEE" service under 37 C.F.R. 1.10, on the date indicated above, and addressed to: Commissioner for Patents, Washington, D.C. 20231.

Jackie Williams
Signature of person mailing page:

Jackie Williams
Type or print name of person

AMENDMENTS

Please amend the application as follows, without prejudice.

In the Claims (Clean Copy):

8. (Amended) Use of a fish feed as claimed in claim 5 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color of the flesh is important, to effect a change in the flesh color.

Please add the following new claims:

10. (New) Use of a fish feed as claimed in claim 6 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color of the flesh is important, to effect a change in the flesh color.

11. (New) Use of a fish feed as claimed in claim 7 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color of the flesh is important, to effect a change in the flesh color.

REMARKS

Claims 1-9 are currently pending. By means of this preliminary amendment, claim 8 has been amended to eliminate a multiple dependency and address some minor formalities. Claims 10 and 11 have been added. The changes are shown in the marked-up copy of the claims that follow this amendment.

It is respectfully submitted that the claims presented in this preliminary amendment are patentable over the art cited during international examination. The prior art does not show the features recited in the claims.

Applicants request early examination of the application on the merits.

If the Examiner believes that direct communication with the Applicants' attorney would advance the prosecution of this application, the Examiner is invited to telephone the undersigned at the number listed below.

Respectfully submitted,

BUTTLE, LOUISE G.

BY: _____

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Marked-Up Copy of Amended Claims

In the Claims (Clean Copy):

8. (Amended) Use of a fish feed as claimed in claim 5 [claims 5 to 7] in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color [colour] of the flesh is important, to effect a change in the flesh color [colour].

Please add the following new claims:

10. (New) Use of a fish feed as claimed in claim 6 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color of the flesh is important, to effect a change in the flesh color.

11. (New) Use of a fish feed as claimed in claim 7 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the color of the flesh is important, to effect a change in the flesh color.

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"PIGMENT"

This invention relates to a method of altering and improving the pigmentation of fish flesh. More specifically the invention discloses a method of enhancing the uptake of pigments by fish, such that there is a resultant increase in the level of pigmentation of the fish flesh.

An increase in market competition, coupled with the widespread availability of fish in supermarkets has led to an increase in the demand for, and quality of fish products.

Mass production of salmonids such as salmon and trout is required to meet current consumer demand which exceeds that which can be met by fish produced in a natural, wild environment.

Variations exist between fish produced naturally and those which are specifically farmed to meet consumer

demand. One particular difference is a variation in the colour of the flesh of the fish.

The characteristic pink colour of salmonid flesh is a result of the deposition of naturally occurring carotenoid pigments. Obtaining pigmentation in farmed salmonids which is similar to that seen in wild salmon is a vital aspect of feed production. Currently fish feeds contain either or both of the main synthetic pigments which are commercially available; astaxanthin (Ax) and canthaxanthin (Cx). In several instances, pigment costs contribute to 10-15% of the total cost of fish feed production, compared to pigment flesh deposition efficiencies which rarely exceed 15%. Since fish feed comprises around 50% of the total production costs of farmed fish, 5-7.5% of overall fish production cost can be attributed to the cost of pigment.

Flesh colour is one of the main criteria used by the consumer when considering the purchase of salmonids and accordingly it is considered by the consumer that the stronger red colour of the flesh which is seen in wild fish is more desirable.

In an effort to achieve the flesh colour characteristics exhibited by wild fish, pigments are added to the feed given to farmed fish with the intent that the uptake, by ingestion of the pigment, will lead to an associated change in the colour of the flesh.

Pigments are specifically selected such that their uptake will lead to the flesh becoming a red colour. Examples of pigments which induce this are canthaxanthin and astaxanthin.

Such processes are not limited to fish, as the modification of the colour of naturally produced foodstuffs is a current trend. The aesthetic appeal of the product to the end consumer is enhanced through modification of the feed ingredients to influence the characteristics of the final product, in particular the colour of the product.

An example of such a process currently known in the art is the alteration of the feed ingredients given to chickens and hens, such that the colour of the yolk of the eggs that are produced is modified from that of the natural colour. The result of this process is that the product has an increased aesthetic appeal which in turn leads to a greater desirability for consumer consumption.

It is desirable for the flesh of the fish to be altered to any specific requirement which may be set. One such method of altering the fish flesh colour would be through the introduction of pigments into the diet.

It is an object of the present invention to provide a method for improving the uptake of pigments which are provided in the diet to influence the colour of fish flesh.

According to the present invention there is provided, a method of enhancing the uptake of pigment by fish, the method comprising feeding fish with cholesterol.

Preferably fish are fed cholesterol and pigment.

Preferably the cholesterol and/or pigment will be a component of the fish feed.

Also preferably the cholesterol will be provided in the same medium as the pigment.

Preferably the cholesterol will be added to the fish feed at a level of between 0.1 to 5 percent.

Most preferably the cholesterol will be added to the feed at a level of between 1 to 3 percent.

Preferably, the pigment will lead to a change in flesh colour, plasma pigment levels and flesh pigment levels of fish.

Preferably the method can be used on Atlantic salmon, rainbow trout, other salmonid species, tropical fish.

Alternatively, the method may be used on any other fish species where the pigment colour of either the flesh or skin is important.

The invention also provides fish feed comprising cholesterol and pigment.

The invention also provides the use of cholesterol to enhance uptake of pigment to fish flesh.

Deposition of carotenoids in the fish flesh occurs as a result of several processes; absorption of pigments in the digestive tract, transport of pigment in the blood, retention in the flesh and metabolism of carotenoids. These processes are further detailed below;

1. **Absorption**

Pigment absorption across the intestinal wall, from the digestive tract to the blood is the initial phase in pigment retention by muscle in salmonids. Since carotenoids are lipid soluble they are most likely to be emulsified in a mixed micelle together with bile, other lipid components, prolipase and lipase during absorption across the gastrointestinal tract (Leger 1985).

The rate of pigment absorption to the blood, following ingestion is fairly slow, compared to the absorption of essential fatty acids and amino acids (Storebakken & No, 1992). Maximum plasma Ax and Cx levels occurred at 24 hours following the force feeding of rainbow trout with a 500mg dose of Ax (March *et al* 1990, Choubert *et al* 1987),

carotenoid levels first being detected at 3 hours following feeding.

2. **Blood Transport**

Ax and Cx are largely transported by the high density lipoprotein fraction of plasma in immature rainbow trout (Choubert *et al*, 1992, 1994). Generally in immature fish, flesh is a major tissue for storing carotenoids (No and Storebakken, 1992).

3. **Deposition/Flesh Retention**

Deposition efficiency of dietary carotenoids in salmonid flesh is in the range 1-18% (Torrissen *et al*, 1989). Astaxanthin retention efficiency of Rainbow trout was found to be significantly higher than that for canthaxanthin; 11.4% and 7.1% respectively (Storebakken & Choubert 1991). Dose response studies show that the efficiency of deposition declines with increase in dietary level (50 mg/kg in Rainbow trout, Storebakken & No 1992: 10 mg/kg in Rainbow trout, Crampton 1995).

Differences in flesh retention efficiencies between species have been observed, and it is known that rainbow trout (RBT) pigment has a greater efficiency than Atlantic salmon (ATS).

In the muscle of wild salmon (*Oncorhynchus keta, O. nerka & O. kisutch*) astaxanthin (90% in the free form) and canthaxanthin are bound to actomyosin, probably *via* weak hydrophobic bonds (Henmi *et al* 1987). Astaxanthin forms two hydrogen bonds per one β ionone ring, and combines more strongly than canthaxanthin, due to its hydroxyl group (see Henmi *et al* 1989). The actomyosin of salmon muscle can associate with many kinds of carotenoids and lipids, implying that specificity of binding sites is not a problem, with variation between molecule types relating to the bond strength (Henmi *et al* 1989). In the skin the majority of astaxanthin is found in the ester form (Torrissen *et al* 1989).

4 Metabolism

Carotenoids and their metabolites have been detected in the tissues of fish up to 96 hours following ingestion of a labelled meal (Guillou *et al* 1992). Schiedt *et al* (1989) found idoxanthin to be a metabolite of astaxanthin in ATS flesh - higher levels of idoxanthin were found in experimental fish in indoor tanks of farmed fish in open cages, which suggests that this may be stress related (Al-Khalifa & Simpson 1988). Metabolites of carotenoids are found mainly in the skin, but also in the flesh of sexually maturing fish (Hata & Hata 1975; Scheidt *et al* 1985).

Schiedt *et al* (1985) evidenced that astaxanthin could be a precursor to vitamin A in vitamin A-depleted fish. Results of Al-Khalifa & Simpson (1988) showed that astaxanthin was converted to zeaxanthin, but in Vitamin A sufficient RBT it was not converted to Vitamin A_1 and A_2 although fish fed a diet lacking in vitamin A and carotenoids for 30 days and then force fed astaxanthin showed an increase in vitamin A.

This document suggests that the incorporation of a pigment into the diet, either in combination with the foodstuffs directly, or as a separate entity introduced into the diet such that it will enter the same metabolic pathways as other ingested and absorbed nutrients will also end up as a constituent of the flesh.

The pigment will lead to a change in the colour of the flesh into which it is incorporated.

The incorporation of the pigment into the flesh may not be efficient and this document identifies a method of enhancing such pigment uptake.

The benefits of a method by which the uptake of pigment by the fish is enhanced are wide-ranging and cover both biological and economical aspects.

The addition of pigments such as astaxanthin and canthaxanthin can have a drastic economical effect on the cost of producing fish feed pellets, due to the expensive cost of the pigments. As such a more efficient mechanism of producing the effects of astaxanthin and canthaxanthin may lead to a reduction in the amount that needs to be added to the feed initially.

Some research has indicated that lipid levels improve pigment absorption for example Choubert et al (1991) found that digestibility of Cx was greatly improved when using a lipid rich diet (14% lipid/dry matter cf 4% lipid/dry matter). However, at commercially realistic levels of lipid (24-35%) no differences were found in flesh deposition efficiencies of RBT (Crampton, 1996 internal data).

Bjerking et al (1997) found no significant effect of dietary protein sources (eg a fish meal against a full fat soyabean meal) in Atlantic salmon fed for 9.5 months, on the amount of astaxanthin in the muscle or the visual colour score.

A study of biological utilisation of carotenoids (α and β-carotene) in rats found that bioavailability of naturally occurring carotenoids was greater than the crystalline form (Tee *et al* 1996). In addition, Bierer *et al* (1995) found that in pre-ruminant calves higher serum levels of carotenoids were observed when given

commercial beadlet sources compared to crystalline sources.

A Patent Application in the name of Finnfeeds International Limited, (WO 9818345 A) claims that the absorption in fish, crustaceans and healthy poultry of pigments present in a non-viscous animal feed is promoted by the presence of a carbohydrase and protease enzyme.

In studies with young chickens Tyczkowski *et al* (1989) found that lipids, long chain saturated fatty acids (myristic, palmitic, stearic) and triglyceride, tristtearin, promoted minimal absorption of lutein, whereas the short chain saturated lauric acid promoted the highest absorption. Screening trials have been conducted to try and identify enhancers of pigment uptake that may be added to the feed to improve pigmentation.

Cholesterol was tested as one of the enhancers, due to its properties as an auxiliary agent in uptake. Cholesterol is an important lipid in some membranes and the plasma membranes of eucaryotic cells are usually rich in cholesterol, this steroid also modulates the fluidity of eukaryotic membranes. Due to these properties cholesterol was identified as a substance with the potential to enhance pigment uptake.

Cholesterol is added to the feedstuffs either by means of extruder or via flex coating with a level of addition between 0.5% and 5%. Natural levels of cholesterol in commercial fish feeds (derived mainly from fish oil) are up to approximately 0.5%.

In the same way that the pigmentation of salmonid flesh, eg Atlantic salmon, Coho salmon, Chinook salmon, Rainbow trout, Artic charr, is important to the consumer, the skin colour of tropical fishes is also an important quality characteristic. In this way the feedstuffs of the above-mentioned species could be modified in a similar way to effect the colour of flesh and skin, in addition to flesh pigment concentration (mg/kg).

A series of experiments are described below which look at whether there is an enhancement of pigment uptake in the plasma and flesh when the fish feed is supplemented with varying levels of cholesterol.

Experiment 1

Atlantic salmon of mean weight 120g, were fed for a period of 72 hours on one of two diets;

Diet 1: contains approximately 40ppm of canthaxanthin (Cx)

Diet 2: contains 40ppm canthaxanthin (Cx) plus 0.48% (total feed, 3% of the lipid coating phase) of cholesterol.

Cx and cholesterol were added in the coating.

After feeding, fish were bled via the caudal vein, using heparanised vacutainers, the blood samples were centrifuged on site and the plasma removed and stored frozen. Plasma pigment levels were analysed on HPLC.

Analysis results for the feeds are shown in Table 1.

TABLE 1 Cholesterol levels in feeds

Fish Feed	Cholesterol addition	% Cholesterol in feed
Uncoated feed	0	0.32
coated feed	0	0.27
coated feed	0.48%	0.53
coated feed	0	0.28
coated feed	0.48%	0.54

TABLE 2 Plasma results for the treatmetns

Replicate	Feed No.	Treatment	Cholesterol level % feed	Feed Cx mg/kg	Plasma Cx µg/ml mean (STD)
1 2	1	CR	0	40.51	0.94 (0.5) 0.64 (0.4)
1 2	2	CR + Cholest-erol	0.48	45.67	1.42 (0.57) 1.45 (0.96)

significant differences were observed $p<0.05$ (T-test)

CR = carophyll red (commercial formulation of Canthaxanthin)

The results shown in Table 2 clearly show that the fish fed with cholesterol in feed (Diet 2) shown almost a 50% increase in the plasma Cx level compared to the control feed. Additionally this trend is apparent in both replicates of the experiments.

Experiment 2

Further experimentation investigating the effect of supplementing dietary cholesterol on astaxanthin and canthaxanthin flesh and plasma levels is described below.

Atlantic salmon of an initial weight of 0.136Kg were grown for four months in 12 x 3m tanks, supplied with seawater. Fish were fed feeds containing varying levels of cholesterol. (Sigma, C8503, approximately 95%). Cholesterol was mixed thoroughly with the oil source and added in the coating (in addition to the pigment preparations of astaxanthin (Ax) and canthaxanthin (Cx)). Soya oil was selected as an oil naturally low in cholesterol and this was the basis for using fish foods with different oil source types and the mixture of oils. Details of dietary cholesterol levels and astaxanthin, canthaxanthin concentrations are given in Table 3.

At the end of the trial, the fish were weighed, they had their blood removed for pigment analysis, and flesh

samples scored with respect to colour and later analysed for pigment.

The results of the experiments are further described with reference to the figures wherein;

Figure 1 shows the effect of feed cholesterol level on flesh pigment (Cx) concentration (mg/kg), with each point on the figure representing the mean value of each of the tanks,

Figure 2 shows the levels of the pigment canthaxanthin in fish flesh, when cholesterol is added to the feed effect of feed cholesterol level (mg/kg), wherein each point is the mean value of each tank (the 5 pooled analyses),

Figure 3 shows the effect of fed cholesterol level on fillet SalmoFan scores and,

Figure 4 shows the effect of feed cholesterol level on Minolta redness (a* value).

Figure 1 shows that the plasma pigment levels show an increase which is correlated with an increase in dietary cholesterol to approximately 1-3%. Any further addition of cholesterol to the feed after this level shows a decline in pigment plasma concentration. Maximum canthaxanthin plasma level values were observed at 3.6 μg/ml (1% feed cholesterol added), compared to control values of 1.5-2 μg/ml.

Figure 2 shows the effects on the levels of the pigment canthaxanthin in fish flesh, when cholesterol is added to the feed. Maximum flesh pigment levels of around 4.3 mg/kg were observed in the group of fish fed canthaxanthin (which have a feed cholesteraol level of 1.3%), compared to levels of around 3 mg/kg in the control groups. In this size of Atlantic salmon, dietary cholesterol levels (1-4%) caused an increase in flesh pigment levels, this increase ranged from 0.4 mg/kg to 1.3 mg/kg.

Astaxanthin flesh levels were 2.32 mg/kg for the control fish and 2.76 mg/kg for the fish with a 3.8% cholesterol supplement to their feed. Astaxanthin plasma levels were 0.62 μg/ml for the control and 0.65 μg/ml for the fish whose feed was supplemented with 3.8% cholesterol.

The effect of increasing the overall percentage of cholesterol in feed with respect to the resultant colour of the flesh is shown in Figure 3. The colour is scored using a Roche SalmoFan™ score. This is a tool used in the industry to score fish colour, which was developed by Hoffman la Roche Ltd. The test comprises a set of different coloured plastic mini sheets which combine to form a scale that ranges from 20 (pale pink) - 34 (dark red), which are used to compare against the colour of the fish flesh and score them accordingly.

Maximum SalmoFan scores were observed with the tanks of fish fed 1-2% cholesterol in the feeds. At higher feed cholesterol levels, a decrease in Roche SalmoFan™ scores was observed (Figure 3). The difference in flesh colour shown by the fish fed diets which had been supplemented with between 1-2% cholesterol related to 1-1.5 points advantage on the Roche SalmoFan™ test.

Further analysis of the flesh colour was carried out using the Minolta evaluation technique. Minolta redness values are shown in Figure 2. The Minolta is a tristimulus colorimeter (Minolta Chroma Meter CR300, Minolta, Japan) which has an 8mm head and a D65 light source. Readings were given for Lightness (L*), Redness (a*) and yellowness (b*), the "L a b" system according to International Commission on Illumination (CIE, 1986). Maximum redness values were observed in the fish fed which been supplemented with between 1-2% of cholesterol, although the pattern was not as clear as that exhibited by the results of the SalmoFan™ scoring system.

In conclusion, although the experiments described herein show that the addition of any amount of cholesterol to a fish feed at the level of 0 to 5% can results in an increase in pigment levels in the plasma and flesh, the results indicate that the optimum uptake of pigment by the plasma and deposition in the flesh occurs when the feed contains a cholesterol level of between 1 to 3% of total feed weight.

CLAIMS

1. A method of enhancing the uptake of pigment by fish to induce a change in the pigmentation of the flesh, said method comprising the step of feeding fish with a feed containing pigment and cholesterol, wherein the cholesterol added to the range of 0.1-5% of the total pellet weight.

2. A method as claimed in claim 1 wherein the feed contains pigment and 0.1-5% cholesterol in the total feed.

3. A method as claimed in claim 1 wherein cholesterol comprises between 1-4% of the feed.

4. A method as claimed in claim 1 wherein cholesterol comprises between 1-3% of the feed.

5. Use of a fish feed containing pigment in the colouration of fish flesh wherein the feed also contains cholesterol at a level of between 0.1-5% of the total feed.

6. Use of a fish feed as claimed in claim 5 wherein cholesterol comprises 1-4% of the feed.

7. Use of a fish feed as claimed in claim 5 wherein cholesterol comprises 1-3% of the feed.

8. Use of a fish feed as claimed in claims 5 to 7 in the feeding of Atlantic salmon, rainbow trout, tropical fish or any other fish species where the colour of the flesh is important, to effect a change in flesh colour.

9. The use of cholesterol in a fish feed, to enhance the uptake of pigment to effect an alteration in the colour of the fish flesh, wherein the level of cholesterol is between 0.1-5% of the total weight of the feed.

1 Table 3:

Feed No	Cholesterol Feed Level (%) Added	Cholesterol Feed Level (%)	Pigment Type	Dietary Pigment Conc (mg/kg)	Oil Source
1441	Control	0.473	Cantha	55.11	fish oil
1442	Control	0.382	Cantha	44.51	Fish/soya oil
1443	Control	0.305	Cantha	50.94	Soya oil
1444	1	1.258	Cantha	46.66	Fish/soya oil
1445	2	2.186	Cantha	50.09	Fish/soya oil
1446	3	3.142	Cantha	52.39	Fish/soya oil
1661	4	4.001	Cantha	50.82	Fish oil
1662	4	3.936	Cantha	53.47	Fish/soya oil
1663	4	3.802	Cantha	48.62	Soya oil
1664	Control	0.412	Asta	47.47	Fish/soya oil
1665	4	3.803	Asta	44.86	Fish/soya sil

2

Figure 1: The effect of cholesterol feed level (%) on plasma cantha level in ATS (μg/ml)

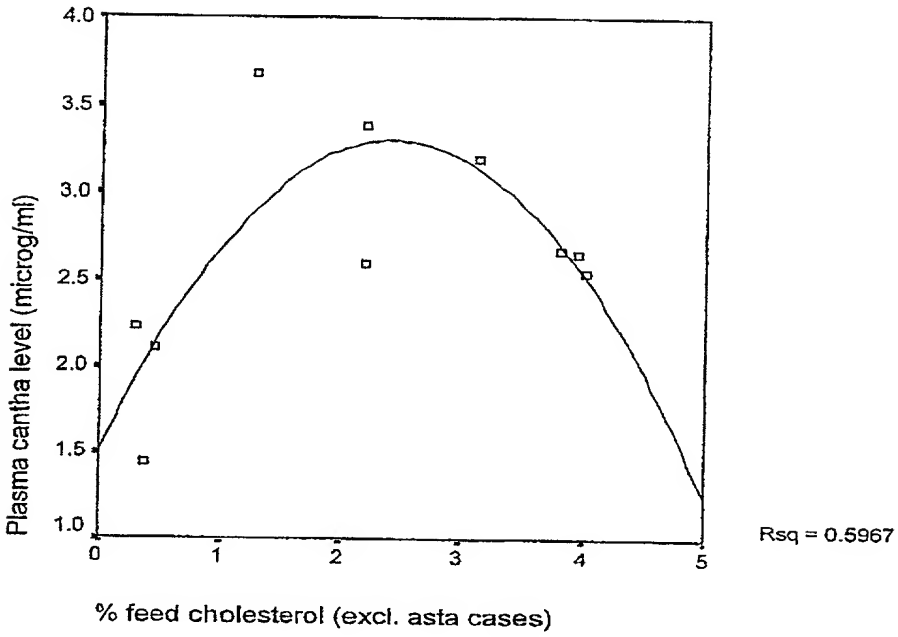

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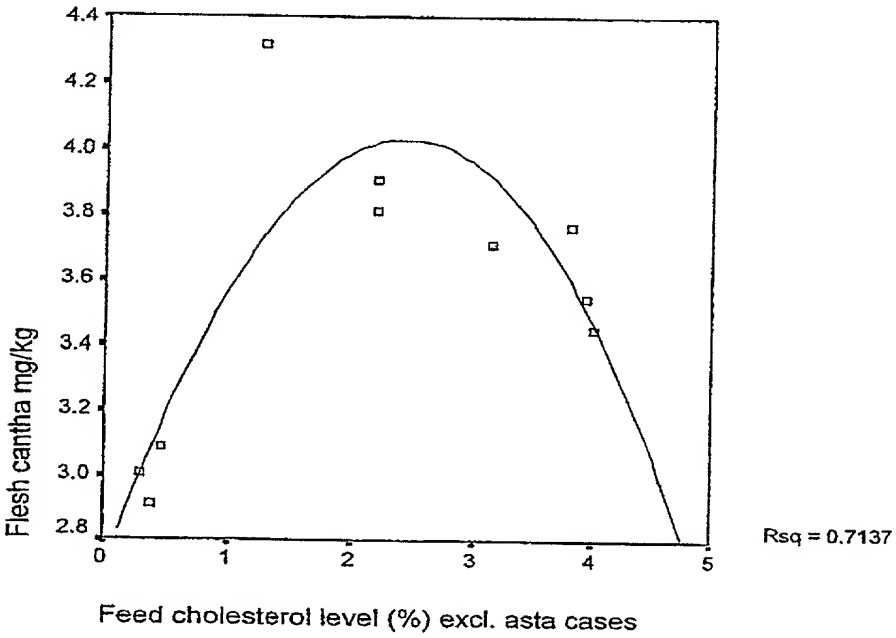
Figure 2: The effect of cholesterol feed level (%) on flesh cantha level in ATS (mg/kg)



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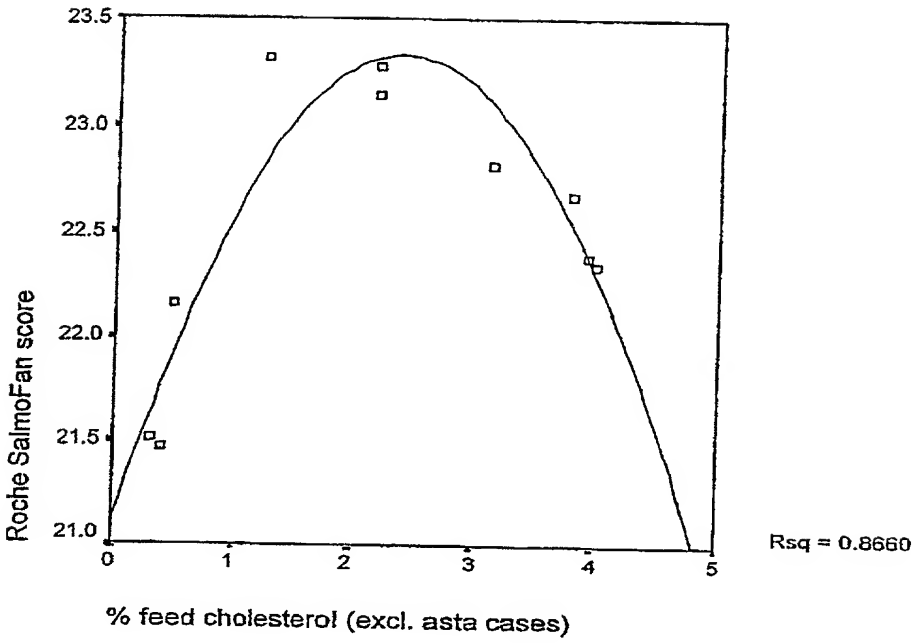

Figure 3: The effect of cholesterol feed level (%) on ~~plasma cholesterol level~~ in ATS ~~(g/l)~~

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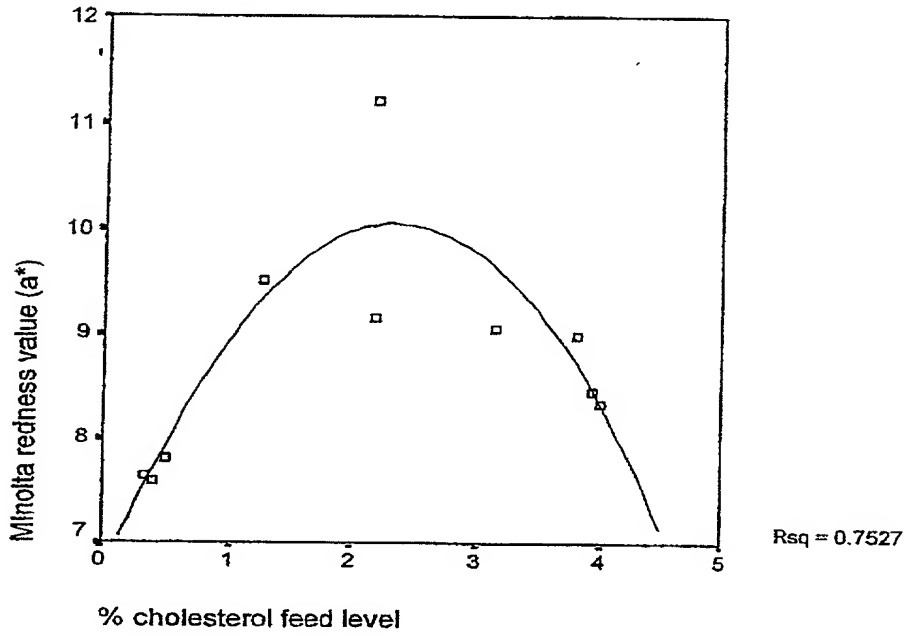
WO 00/67591 PCT/GB00/01753

Figure 4: The effect of feed cholesterol level on minolta redness (a*) value



DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are stated below next to my name:

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

PIGMENT

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the specification of which was filed on May 8, 2000 as PCT Application No. PCT/GB00/01753 and amended on May 15, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with 37 CFR §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed:

PRIOR FOREIGN/PCT APPLICATION(S)

COUNTRY/OFFICE	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
United Kingdom	9910461.4	May 7, 1999	☒YES NO ☐

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	DATE OF FILING
None	
(Application Number)	(Filing Date)

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose material information as defined in 37 CFR §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S. FOR BENEFIT UNDER 35 U.S.C. §120

Application Serial No.	Date of Filing	Status (check one) Patented	Pending	Abandoned
NONE		☐	☐	☐

And I hereby appoint Arthur H. Seidel, Registration No. 15,979; Gregory J. Lavorgna, Registration No. 30,469; Daniel A. Monaco, Registration No. 30,480; Thomas J. Durling, Registration No. 31,349; John J. Marshall, Registration No. 29,671; Joseph R. DelMaster, Jr., Registration No. 38,123; and Robert E. Cannuscio, Registration No. 36,469, my attorneys or agents with full power of substitution and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

Address all correspondence to **Robert E. Cannuscio**, Drinker Biddle & Reath LLP, One Logan Square, 18th and Cherry Streets, Philadelphia, Pennsylvania 19103-6996. Address all telephone calls to **Robert E. Cannuscio** at 215-988-3303 (telefax: 215-988-2757).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

FULL NAME OF FIRST INVENTOR

LOUISE	**GEORGINA**	**BUTTLE**
(GIVEN NAME)	*(MIDDLE INITIAL OR NAME)*	*(FAMILY OR LAST NAME)*

Inventor's signature:______________________________

Date:______________________________

Country of Citizenship: Great Britain

Residence: Edinburgh United Kingdom

(City) *(State or Foreign Country)*

Post Office Address:

78 Harrison Gardens
Edinburgh, United Kingdom
EH11 1SB

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LOUISE	**GEORGINA**	**BUTTLE**
(GIVEN NAME)	*(MIDDLE INITIAL OR NAME)*	*(FAMILY OR LAST NAME)*

Inventor's signature:____

Date:____

Country of Citizenship: Great Britain

Residence: Edinburgh (*City*) United Kingdom (*State or Foreign Country*)

Post Office Address:
78 Harrison Gardens
Edinburgh, United Kingdom
EH11 1SB

PATENT
Attorney Docket No. 8830-10 (157952)

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LOUSIE	~~**GEORGINA**~~	**BUTTLE**
(GIVEN NAME)	*(MIDDLE INITIAL OR NAME)*	*(FAMILY OR LAST NAME)*

Inventor's signature: [signature]

Date: 30-11-01

Country of Citizenship: BRITAIN

Residence: PUERTO VARAS CHILE

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